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Evaluation of Mixed Exposure to Carcinogens and Correlations of *In Vivo* and *In Vitro* Systems

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The biological evaluation of air pollutants is an example of the difficulties of evaluating the effects of mixed concurrent exposures to multiple agents, such as combinations of carcinogens with other carcinogens of the same or different chemical class, with incomplete carcinogens and cocarcinogens, with particulate materials and other factors that modify tissue distribution and retention, and with modifiers of metabolic pathways of activation and detoxication.

A research approach is outlined to investigate such interactions in a series of biological systems of increasing complexity but closely related to each other in a step-by-step sequence, e.g., bacterial mutagenesis; mammalian cell mutagenesis, toxicity and neoplastic transformation, including embryo cells, fibroblasts and epithelial cells; organ cultures of target epithelia; *in vivo* animal systems for short-term and long-term studies, including animal models closely comparable to human pathology; observational studies of human pathology and histopathogenesis; experimental studies of corresponding human target tissues using organ and cell culture methods for metabolism, toxicity, mutagenicity and possibly neoplastic cell transformation.

Respiratory carcinogenesis models were successfully used for studies of mixed exposures to different carcinogens and cofactors. The role of particulates has been found to be important but needs to be further characterized.

Quantitative variations in the response to carcinogens and cofactors among different biological test systems and among different individuals in the human population make quantitative risk estimation very difficult, but studies in a sequence of related biological systems including human tissues indicate the importance of qualitative risk evaluation.

Biological Evaluation of Mixed Exposures to Different Carcinogens, Including Air Pollutants

The biological evaluation of the air pollutants under consideration—motor exhausts and coal combustion products—is a representative example of the difficulties encountered in evaluating the effects of mixed concurrent exposures to multiple agents. These mixed exposures include the following categories of combinations of factors:

(a) Carcinogens of the same chemical class, e.g. different polycyclic aromatic hydrocarbons (PAH)

(b) Carcinogens of different chemical classes, e.g. PAH, nitrosamines, halogenated hydrocarbons, metal compounds

(c) Complete carcinogens and incomplete carcinogens, e.g. initiators, promoters and cocarcinogens

(d) Carcinogens and factors that modify carcinogens issue distribution, retention and response conditions, e.g. particulate materials

(e) Carcinogens and cofactors in the exposure source combined with other exogenous and endogenous carcinogenic factors

(f) Carcinogens and modifiers of their metabolic pathways of toxication and detoxication

The problem of evaluating multiple exposures to different carcinogenic factors has not been investigated very extensively so far because of its intrinsic complexity. Yet, all carcinogenic exposures in the

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human population occur in the context of a wide background of other exposures to different carcinogens and to response modifiers.

Increasing recognition is being given to the multifactorial origin of human cancers. This concept implies that any particular type of exposure under consideration has to be evaluated in the context of a number of other factors that can all contribute in different proportions to the causation of cancers in a population. In proposing a model for such multifactorial interactions, I have suggested (*1,2*) that the following broad categories of factors be considered for their contribution to the multifactorial origin of cancer:

- Categories of factors deemed to be intrinsic components of the causative complex of all cancers and each therefore involved in 100% of cases; these categories include genetic factors, nutritional factors and unknown factors;
- Categories of factors considered as present in the history of practically all cancers but likely to contribute to a causative role only in a portion of the cases; these categories include physical factors, inhaled factors, as well as dietary and other environmental contaminants;
- Categories of factors considered to be present only in a portion of the cases and, within that portion, to be more or less effective as causative agents: these categories include non-genetic hormonal and sexual factors (in a range of 20-40% of cases), infectious agents (in a range of 1-3% of cases), and occupational factors (in a range of 20-40% of cases).

Other factors are sometimes considered as separate groups in other classifications, such as socioeconomic, cultural and "lifestyle" factors, but in view of their ill-defined nature they are included in the category of unknown factors in the proposed model.

The range of relevance of each category of factors is therefore at or near 100% for several categories and less than 50% for others. The "sum" of the concurrent contributions of all factors is over 600%. If one considers the number of individual carcinogens present in some of these categories of exposure, e.g., inhaled factors or environmental contaminants, one can visualize a much larger layering of overlapping concurrent exposures, each potentially contributing a share, however small, to the total carcinogenic exposure burden. Under these conditions, the overlap of the composite "sum" of the participating exposures can be well over 1000%.

Any number of combinations can be visualized in the interplay of the different factors towards the causation of cancer in a population, the intensity of the causative contribution of each factor being represented by a wide range, from a very prominent level to a very low level that may become indistinguishable from mere exposure without effect (*1,2*).

To distinguish mere exposures without a causative effect from exposures followed by an effect in the process of cancer causation is a singularly difficult problem if the effect is not a prominent one but constitutes a small contribution to the multifactorial complex of factors that all together result in a given cancer frequency in a population. The contribution of an individual carcinogen to the total causative complex may be completely below its level of detectability by common bioassay methods, let alone by the relatively insensitive epidemiologic methods, when that carcinogen is acting as a component of a large number of exposures including many other different factors.

As a realistic model for the complex multifactorial aspects of carcinogenesis in a heterogenous human population one could consider a bioassay comparing groups of animals, each made up of mixed populations of many different inbred and noninbred strains, not controlled for diet and other general conditions, with various intercurrent diseases, exposed by an inconsistent schedule to many different carcinogens and cofactors. Let us suppose that we had two such heterogeneous groups of animals, each exposed at various times to about 100 different carcinogens, but not quite the same carcinogens nor at the same doses in the two groups: to these complex exposures we then add a given test compound (the 101st exposure) in one group but not in the other. The likelihood of obtaining any meaningful finding in such a comparison is obviously scanty, unless the single test compound has very uncommon properties and shows a very high level of activity. Even so, its demonstration in the presence of so many confounding variables would be highly questionable. Such a situation, that appears experimentally so absurd as a model for cause-and-effect studies, unfortunately represents an already simplified model in comparison with most human population studies on the exposure to single agents.

The patterns of interaction among carcinogens that take place during concurrent multiple exposures are generally little known, except for the important aspect of the regulation of carcinogen metabolism through the induction of carcinogen metabolizing enzymes by xenobiotics (including other carcinogens): even here, however, the balance of metabolic activation and detoxication in a complex exposure system is often difficult to predict. Different biological models also show considerable variation in this respect. Differences in

genetic make-up, in age and in nutritional conditions lead to considerable variations in the type and level of response to carcinogens, making it therefore difficult to extrapolate risk estimations from any one experimental system to the human.

A special research approach is needed to extricate ourselves from these complex difficulties and to identify those recognizable patterns in the interacting effects of multiple carcinogens that could provide us with an understanding of the carcinogenic risks from complex exposures in the human.

An approach that I consider particularly promising in this respect consists of the study of interactions of carcinogens in a series of biological systems of increasing complexity but fairly closely related to each other in a step-by-step sequence, linking together the response of simple and well defined unicellular targets in well controlled microenvironments to the response in organized target tissues, organs and whole organisms, including not only animal pathology but also human pathology. Such an approach initially extends over a long period of time, since it requires the development of a wide range of biological models related to each other and ultimately to human cancer pathology. Fortunately a great deal of progress has occurred in this direction in the past two decades through major advances in experimental pathology, cell biology and biochemistry methods.

A systematic investigative approach is therefore becoming possible to study the interactions of selected categories of carcinogens in various combinations in a sequential series of biological target systems, both *in vivo* and *in vitro*.

A suitable series of systems can include the following components:

- Bacterial mutagenesis systems
- Mammalian cell mutagenesis systems
- Mammalian cell systems for the analysis of toxic effects
- Mammalian cell systems for neoplastic transformation, either in embryo cells and fibroblast cell lines or in specialized epithelial cells (e.g., epidermis, liver, respiratory epithelium)
- Organ explant culture systems for target epithelia with the possible outgrowth of epithelial cell lines
- *In vivo* animal systems for short-term and long-term studies on target epithelial tissues and organs, including animal models for carcinogenesis closely comparable to their human pathology counterparts
- Observational studies of histopathogenesis and cell differentiation in human pathology, compared with the animal systems listed above.

Advances in the methods for the culture of human tissues and cells (*3*) have recently made it possible to extend the series to the following experimental human counterpart systems:

- Organ culture systems for human target tissues
- Cell culture systems for human target cells, particularly epithelial cells for their possible transformation
- Human cell culture systems for mutagenesis and toxicity studies

Many of these biological model systems are already well established, while others are still in the process of development, such as the induction of neoplastic transformation in human epithelial cells in culture by chemical carcinogens.

Chemical induction models *in vivo* have been established in animal systems for most of the major forms of human cancers: they include models for epithelial cancers (e.g., epidermis, bronchus, larynx, esophagus, glandular stomach, colon and rectum, pancreas, liver, biliary passages, kidney, bladder, mammary gland, endometrium, uterine cervix, ovary, prostate, thyroid and other endocrine organs), as well as models for nonepithelial neoplasia (e.g., lymphomas, leukemias and neoplasms of the nervous system and of connective tissues).

Epithelial cell culture systems have been recently established in several laboratories for the corresponding tissues of origin, both from the experimental animal models and from human tissues (e.g., epidermis, trachea/bronchus, lung, esophagus, colon, pancreatic duct, kidney, bladder, mammary gland, endometrium, uterine cervix and prostate).

Methods for the chemical induction of neoplastic transformation in epithelial cells in culture have also been established for animal cells of different tissues (e.g., liver, epidermis, tracheobronchial epithelium, bladder).

Advances in these fields were the subject of extensive recent reviews (*3,4*).

Systematic studies can now be designed in a relevant sequence of biological model systems to investigate combined effects of representative different carcinogens and their mixtures. Metabolic pathways of carcinogen activation and detoxication as well as types of morphological and biochemical responses can be comparatively studied in these sequential series of biological models to identify those patterns that remain constant through the series, up to the more complex biological systems and to human tissues, thus providing a wide biological and comparative basis for the evaluation of their mechanisms and their potential risks as they relate to specific target effects.

Respiratory Carcinogenesis: Evaluation of Mixed Exposures in Model Systems *In Vivo* and *In Vitro*

Respiratory carcinogenesis, particularly in regard to air pollutants, provides a particularly important field of investigation for combined effects of multiple exposure factors.

The experimental animal models for respiratory cancer induction, which have been demonstrated most closely to resemble their human counterparts, i.e., the bronchogenic carcinomas, are those obtained with combined administrations of carcinogens and suspended particulate materials such as metal oxides (*5–12*) or by combinations of these mixed treatments with other topical (*13*) or systemic carcinogens (*14,15*).

An important aspect of the study of mixed exposures in respiratory carcinogenesis is represented by the role of particulate matter. A highly effective experimental method for the induction of bronchogenic carcinomas was developed by intratracheal administration in the hamster of saline suspensions of carcinogens, notably benzo(a)pyrene, carried by fine particles of nonfibrogenic inorganic materials such as ferric oxide (*5*). This method was widely used to investigate the conditions under which the mixed exposure to carcinogens and particulate materials is most effective in producing a high level of carcinogenic response from the respiratory tract epithelium, closely comparable to human bronchogenic carcinoma. The physical characteristics of the carcinogen-particulate preparations were found to be important in determining the retention rate of the carcinogen in the respiratory tract and the level of carcinogenic response (*7,16, 17*).

The physical conditions that contribute to these combined effects are also complex, involving not only the particle size of the carrier dust, but also the particulate state of the carcinogen itself and its physical relationship to the surface of the carrier particles (*18–23*).

Different types of carrier particles have been found effective in enhancing respiratory carcinogenesis by polycyclic aromatic hydrocarbons, including ferric oxide (*5*), titanium oxide (*24*), magnesium oxide (*25*), carbon (*24*) and iodine (*26*).

The interaction of particulate matter in respiratory tract carcinogenesis has been found to occur even in combination with systemically administered carcinogens, as was shown in hamsters by SC injections of diethylnitrosamine followed by intratracheal instillations of ferric oxide particles suspended in saline which markedly enhanced the incidence of peripheral lung tumors (*14*). Repeated intratracheal instillations of saline alone in such a system (i.e., following diethylnitrosamine pretreatment) may also result in a higher level of peripheral lung tumor response, suggesting a nonspecific effect probably mediated by enhanced cell proliferation in the peripheral lung. Progress in the development and study of respiratory carcinogenesis models was extensively reveiwed by Nettesheim and Griesemer (*27*).

The establishment of organ and cell culture methods for respiratory epithelia was the major step towards a more detailed investigation of carcinogenesis mechanisms at the metabolic and cellular level. These methodological developments and their implications were reviewed in recent years (*27–29*) and suggest that there is now available a wide choice of interrelated biological systems for the study of respiratory epithelia *in vivo* and *in vitro*, including tissue explants and isolated epithelial cell cultures from both animal and human sources.

While considerable progress has been made in the elucidation of metabolic pathways of carcinogen activation in target respiratory tissues, including the identification of the specific DNA adducts of several carcinogens administered individually, very little work has been devoted so far to studies on the mechanisms of combined effects of multiple exposures to different carcinogens in these systems.

It is our intent to develop such studies in the new facilities assigned to our Laboratory at the National Cancer Institute's Frederick Cancer Research Facility.

Relevance of Respiratory Carcinogenesis Methods to the Evaluation of Risks from Complex Exposures

The further development of extensive experimental observations on the effects of complex exposures in a sequential series of biological systems closely related to the human respiratory target epithelium will have the considerable advantage to make it possible to correlate the findings from biochemical and morphological mechanism studies from animal species to the human counterpart at the tissue and cellular level, *in vivo* and *in vitro*. A particular difficulty for quantitative comparisons on the effect of carcinogenic exposures between animal studies and their human counterpart is represented by the fact that the level of response elicited by carcinogen exposures in human

tissues has been found to have a wide range of quantitative interindividual variability as indicated by several measurable parameters such as carcinogen metabolism, carcinogen–DNA binding or human tissue-mediated mutagenesis (*31*).

A critical evaluation of human carcinogenic risks requires careful definition of both sides of the host–environment interaction. The "host factor" has to be characterized in terms of genetic susceptibility, metabolic competence, nutritional state and also in terms of exposures to any other relevant environmental contributing factor. The "exposure factor" also needs to be carefully characterized from both the physical and the chemical points of view. The possible interactions of different components of an environmental mixture need to be defined in relation to the experimental conditions used in biological evaluations. For example, as indicated above, variations in the particle size distribution of test materials can greatly influence their distribution, persistance and biological effects in the respiratory tract.

Sampling methods and characterization of test samples need to be accurately defined and reported. Reproducibility of the induced biological effects is a good indicator of the reliability of the findings if they are obtained with samples that are prepared independently.

Certain types of interactive effects of the components of complex mixtures may be dependent on specific exposure conditions. For example, the prominent role of particular matter in determining the level of carcinogenic response *in vivo* to respiratory carcinogens may be in part dependent on the specific hyperplastic effects induced in the respiratory epithelium by the administration of particulates; we do not know yet if a similar effect occurs in tests for neoplastic transformation conducted on isolated cell systems. We have found a marked mutual inhibition of mutagenic activity in the Salmonella assay among several polycyclic aromatic hydrocarbons and their mixtures, while certain other carcinogens were found to enhance the mutagenic activity of benzo[*a*]pyrene in this assay (*30*). We do not know yet whether these effects will persist in biologically more complex systems. These questions can be investigated in the sequential series discussed above using defined prototype preparations of multiple components to determine whether a given combined effect is triggered at the metabolic activation level, outside or inside the target cells, or at the level of genotoxic changes, or at the level of cellular or tissue responses or perhaps only at the organ level because of special anatomical and functional interactions.

Although a number of important investigations of mutagenic and carcinogenic effects of complex mixtures related to air pollution exposures have been already conducted in several biological systems, as shown by the documentation assembled for this conference, there have not been any systematic attempts yet to correlate the effects of mixed exposures through a sequence of biological systems ultimately related to the human target tissues and cells. It is hoped that this conference will stimulate further efforts in this direction.

Risk assessment has become a widely studied subject. It has been pointed out, however, that risk evaluation need not and often should not be forced into the rigid framework of numerical risk estimates: the most important and most critical aspect of risk evaluation may still be the qualitative one, especially in its relation to the comparability of induced effects in the test systems and in the corresponding human situation.

These aspects, correlating progress in experimental pathology with the problem of risk evaluation, were recently reviewed (*1,2,29,32–34*). It is hoped that current and future progress in experimental pathology studies of carcinogenesis mechanisms will contribute to a much more precise understanding of the effects of mixed exposures to carcinogens and their cofactors.

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